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Generation and Evaluation of an Anti-REV1 Monoclonal Antibody

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ABSTRACT

Continuous exposure of cells to exogenous and endogenous agents produces many types of DNA damage during normal cell cycles. Post-replication repair, consisting of error-free and error-prone sub-pathways, is required for tolerance of such DNA damage. REV1 plays a crucial role in regulation of the error-prone pathway. To facilitate analysis of its cellular functions, we here generated a mouse Rev1 monoclonal antibody, called D6, which also recognizes human REV1. The epitope for the antibody could be mapped between 860-877 amino acid residues of human REV1, which was located outside of the conserved catalytic domain. Although the antibody unfortunately could not specifically detect endogenous mouse and human REV1 by western blotting and immunohistochemistry, we established a method to identify endogenous human REV1 by immunoprecipitation-western blotting analysis combining D6 and separately generated polyclonal antibodies.

Key words: *Translesion DNA synthesis, REV1, Antibody*

Continuous exposure of cells to exogenous and endogenous agents produces many types of DNA damage. Lesions of a template strand, which block replication machinery, could pose serious problems for cell survival. However, to rescue such stalled replication, cells have evolved mechanisms, which are known collectively as postreplication repair[5)], comprising error-free and error-prone sub-pathways. REV1, REV3, and REV7 are believed to be involved in the error-prone pathway[11)]. REV1, a deoxycytidyltransferase, incorporates dCMP opposite template apurinic / apyrimidinic sites[12,13,16,17,20,24)] and has a role in DNA damage-induced mutagenesis[7,11)]. It has been categorized as a member of the conserved Y family of DNA polymerases (pols), including pol η, pol ι and pol κ [23)]. REV3 encodes a catalytic subunit of pol ζ [21)] while REV7 is a non-catalytic subunit of pol ζ [19,21)]. These pols are generally referred to as translesion DNA pols because of their capacity for lesion bypass replication[5,11)].

In addition to its catalytic activity, REV1 plays a crucial role in regulation of the error-prone pathway. It has a domain at the C-terminus which can interact not only with all other translesion DNA polymerases, pol η, pol ι, pol κ and pol ζ, (REV3-REV7 complex) but also with REV7[2-4,8,9,15,18,19,22,26)]. The interaction between REV1 and REV7 is stable, with formation of REV1-REV7 heterodimers[4,15)]. Interestingly, this process prohibits interaction with translesion pols[4,8,18,22)].

Recently, it has been proposed that REV1 plays distinct roles in the S and G2 phases in DT40 cells[6)]. Notably, in yeast the amount of Rev1 protein is cell cycle-regulated and is maximal at the G2/M interphase[27)]. Information on human REV1 is crucial for understanding its cellular functions and analysis of cellular REV1 proteins with anti-REV1 antibodies can greatly facilitate this understanding. Here, we report the generation of an anti-REV1 monoclonal antibody and its application.

MATERIALS AND METHODS

Cell culture

A human embryonic kidney epithelial cell line expressing the simian virus 40 (SV40) large T

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antigen, 293T, and a SV40-transformed human fibroblast cell line, GM02063[25], were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO) and 1% penicillin/streptomycin (Sigma). HeLa cells were cultured in DMEM (Sigma) supplemented with 10% heat-inactivated newborn calf serum (NBCS, Sigma) and 1% penicillin/streptomycin (Sigma).

Antibodies

Hybridoma cells were generated from mice, which had been immunized with his-tagged mouse and human REV1 as described[10]. A hybridoma producing an anti-Rev1 antibody, D6, was expanded in culture, and antibody-rich supernatants were concentrated by ammonium sulfate precipitation, then purified by serial column chromatography using hydroxylapatite (Bio-Rad) and Mono Q (GE Healthcare). The immunoglobulin subclass of D6 was determined as IgG1-κ. A polyclonal antibody against a truncated human REV1, consisting of 1-153 amino acid residues containing the BRCT domain, was also raised in a rabbit[15]

Proteins

His-tagged mouse[16] and human REV1[17], including deletion derivatives[14,17,24], and untagged human REV[13], were purified as described earlier.

Immunoprecipitation (IP) with D6

Cell pellets (1×10^7 cells) were suspended in lysis buffer (500 µl), consisting of 20 mM Hepes-NaOH (pH7.9), 420 mM NaCl, 25% glycerol, 0.1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 0.2% NP-40, and 1 × Complete Mini EDTA-free Protease inhibitor cocktail (Roche), incubated for 30 min on ice and centrifuged at 13,000 rpm for 10 min at 4°C. Cell lysates were incubated with Protein G-Sepharose (10 µl of a 50% slurry) (GE Healthcare) for 1hr at 4°C and, after a brief centrifugation, the supernatants were incubated with the anti-REV1 monoclonal antibody (1 µg) or a mock antibody (mouse IgG1-κ; Sigma) overnight at 4°C. Thereafter, Protein G-Sepharose (10 µl of a 50% slurry) was added and incubation continued for 2 hr at 4°C. Immune complexes bound to Protein G-Sepharose were washed five times with lysis buffer then analyzed by western blotting.

Transfection of short interfering RNA (siRNA) duplexes

Ribonucleotide siRNA duplexes for REV1, GCAUCAAAGCUGGACGACUtt (ID #117970) and CCAGUAAAUGGCUGUAAUAtt (ID #117971), and GAPDH, and a negative control were purchased from Ambion. 293T and GM02063 cells were transfected with siRNA using siPORT Amine Transfection Agent (Ambion) according to the manufacturer's instructions. To determine the cell viability, both floating cells in the medium and viable cells on the plates were collected. After staining with trypan blue dye, both viable (unstained) and dead (stained) cells were counted with a hemocytometer, to obtain the percentage of viable cells.

RESULTS AND DISCUSSION

Generation of a monoclonal antibody against mouse and human REV1

To generate anti-mouse and anti-human REV1 antibodies, mice were immunized with his-tagged recombinant mouse and human REV1 proteins, produced in *Escherichia coli* cells and purified by Ni-chelating chromatography. After screening of hybridoma cells, a hybridoma, which produced an antibody, D6, was obtained from a mouse immunized with mouse Rev1. Fortunately, we found that D6 could detect not only mouse Rev1 but also human REV1, as demonstrated by western blotting using purified mouse and human REV1 (Fig. 1A). To determine the region containing the epitope for D6, a series of deletion derivatives of recombinant human REV1[24] were tested with D6 by western blotting. The result demonstrated that the 847-885 amino acid residues of human REV1 were required for recognition (data not shown). For further precision, we performed competition assays using three synthetic peptides, D6-1, D6-2 and D6-3, which were partially overlapping and corresponded to residues 851-868, 860-877 and 869-886, respectively (Fig. 1B). Western blotting results demonstrated that D6-2 reduced the signals most efficiently in a concentration-dependent manner, suggesting competitive binding of D6-2 to D6 under the conditions used. Therefore, we concluded a location for the antibody epitope within amino acid residues 860-877 of human *REV1*.

Evaluation of properties of the D6 monoclonal antibody

First, we tested whether D6 could specifically recognize endogenous REV1 protein by western blotting and immunohistochemistry. Unfortunately, the results showed that D6 could recognize many cellular proteins, and we could not identify the REV1 protein by either method even when the expression level of the REV1 gene was reduced by siRNAs (data not shown), indicating a non-specific nature.

Next, we performed immunoprecipitation (IP)-western blotting analysis using our two different antibodies. A lysate of HeLa cells was reacted with D6 and immunoprecipitated, then the precipitated materials were subjected to western blotting probed with polyclonal antibodies against the BRCT domain of human REV1. Note that the polyclonal antibodies also could not specifically recognize the endogenous REV1 protein by west-

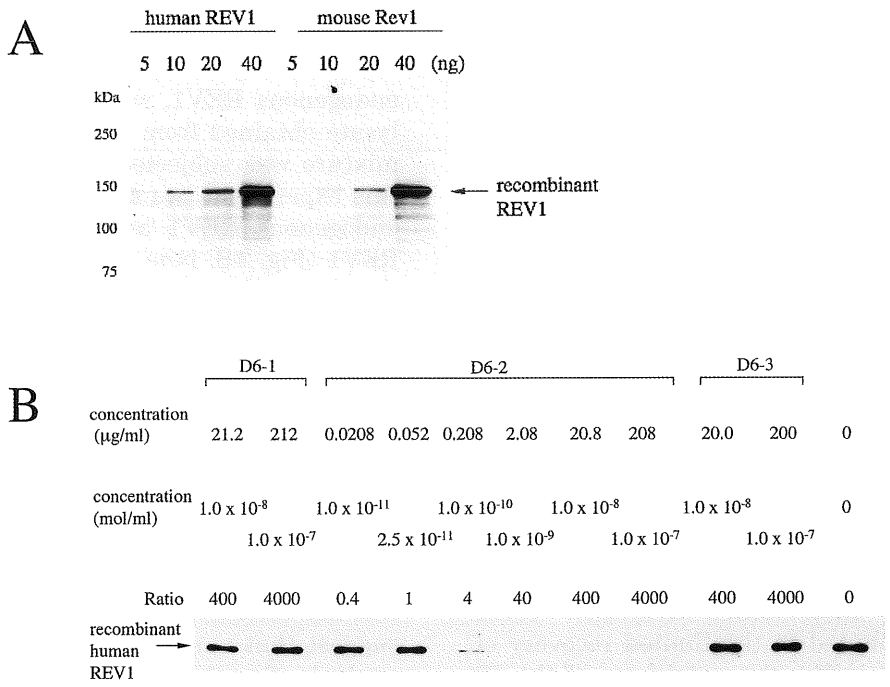

Fig. 1. Epitope mapping for our anti-REV1 monoclonal antibody, D6
(A) The anti-REV1 monoclonal antibody detects both human REV1 and mouse Rev1. Indicated amounts of recombinant proteins were used for western blotting. (B) Competition assays with synthetic peptides. D6 was pre-incubated with the synthetic peptides, D6-1, D6-2, and D6-3, for 2 hr at room temperature before western blotting. Molar ratios of the peptides to the D6 are shown. The amino acid sequences of the three peptides were as follows: SVRDVFQVQKAKKSTEEE (D6-1), KAKKSTEEEHKEVFRAAV (D6-2), and HKEVFRAAVDLEISSASR (D6-3).

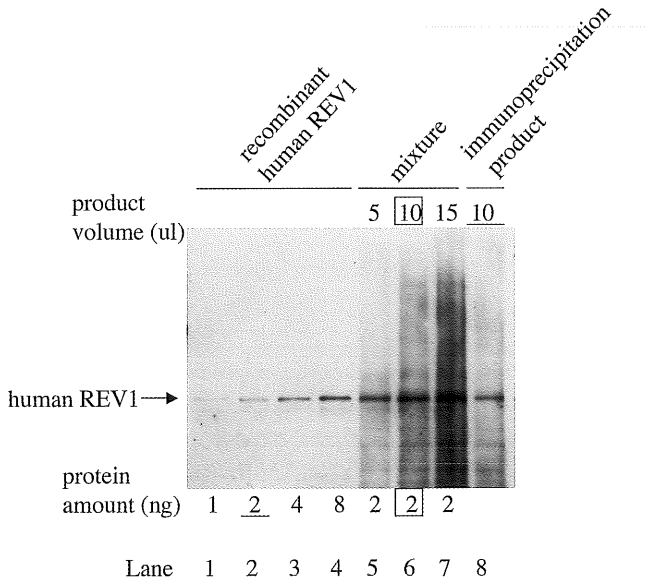

Fig. 2. Specific detection of cellular REV1 by IP-western blotting using two different antibodies.
The indicated amounts of IP products with D6 from HeLa cell lysates and recombinant REV1 were loaded alone or together as mixtures to compare sizes. Lanes 1-4, recombinant REV1; lanes 5-7, mixtures of the IP products and recombinant REV1; lane 8, IP products.

ern blotting. However, IP-western blotting analysis using the different antibodies could reduce the non-specific signals. As expected, the experiment revealed a specific signal with exactly the same size as untagged recombinant human REV1 protein (Fig. 2). When the IP products with D6 and the recombinant human REV1 were mixed together and subjected to western blotting, the signal was still detected as a single band (Fig. 2, lanes 5, 6, and 7) with enhanced intensity (Fig. 2, compare lanes 6 and 8). These results suggested that the detected protein in HeLa cells was identical to recombinant REV1.

To further confirm that the detected signals were from REV1 protein, expression of REV1 was down-regulated by RNA interference (RNAi) technology. First, we confirmed that two different siRNAs efficiently reduced REV1-mRNA levels in GM02063 and 293T cells, as ascertained by RT-PCR (data not shown). Analysis of the cell lysate by IP-western blotting then demonstrated that the intensity of the signals was significantly reduced at 12, 24, 48, 72, and 96 hrs after transfection (Fig. 3A, B). Taking all of the results together, we concluded that the signal detected by IP-western blotting was from REV1.

Estimation of recovery of REV1 by IP-western blotting

Recently, the amount of REV1 protein was estimated to be approximately 60,000 molecules in a single human cell[1]. To estimate recovery of REV1 by IP-western blotting, a lysate prepared from 1 × 10^7 HeLa cells, which contained approximately 2 mg protein, was subjected to analysis with recombinant REV1 protein as a standard. The results demonstrated that the intensity of signals for endogenous REV1 by IP-western blotting corresponded to about 10 ng of the recombinant REV1 (Fig. 4A). Consequently, we could calculate that the amount of REV1 recovered from a single HeLa cell was about 4,000 molecules. This estimation was based on three independent experiments. In addition, essentially identical results were obtained with 293T and GM02063 cells (data not shown). The fact that the amount of REV1 protein was much lower than 60,000 molecules[1] could probably be attributed to the limited recovery of REV1 in lysates, and/or the sensitivity of the IP reaction. To determine the recovery of REV1 on IP, 10 ng of a deletion mutant of REV1, C885, consisting of 1-885 amino acid residues[17] with the C-terminal part truncated for distinction from endogenous REV1, was mixed with 2 mg of cell lysate obtained from 1 × 10^7 HeLa cells, and the mixture was subjected to IP-western blotting. In this experiment, we detected two signals, one for endogenous REV1 and the other for truncated REV1 (Fig. 4B, lane 1 and 2). On comparison of the IP signals with a standard (Fig. 4B, lanes 3-6), we could estimate that 5 ng of the recombinant REV1, 50%, was recovered after IP. Note that the IP reaction was already saturated, since reactions with both 0.4 and 4 μg of antibodies resulted in the same intensities of staining of REV1 proteins (Fig. 4B, lane 1 and 2). Taken together, we could estimate the recovery of REV1 by IP-western blotting to be 7%, and the recovery of REV1 in the lysate to be only 14%. The majority of REV1 (86%) was not extracted by the procedure. Therefore, we suggest that our method might facilitate specific analysis of a soluble fraction of cellular REV1 protein.

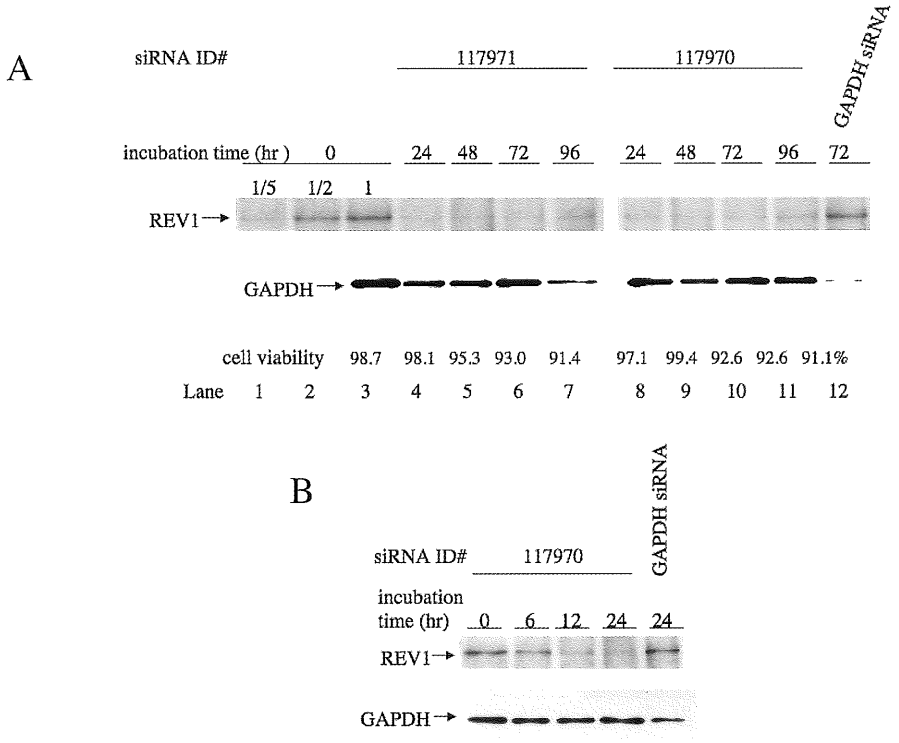

Fig. 3. Knockdown of human *REV1* by siRNA.
(A) IP-western blotting with siRNA treated 293T cells. The cells were transfected with 10 nM *REV1*-specific siRNA (ID #117970), 30 nM *REV1*-specific siRNA (#117971), and 10 nM of *GAPDH* siRNA. As a standard, reduced amounts of cell lysate to 1/5 and 1/2 were subjected to IP-western blotting (lanes 1 and 2, respectively). *GAPDH* siRNA, not related to REV1 expression, was used as a negative control (lane 12). Viability of cells at each time point after treatment with siRNAs is shown. (B) Samples of different time points were analyzed as shown in A.

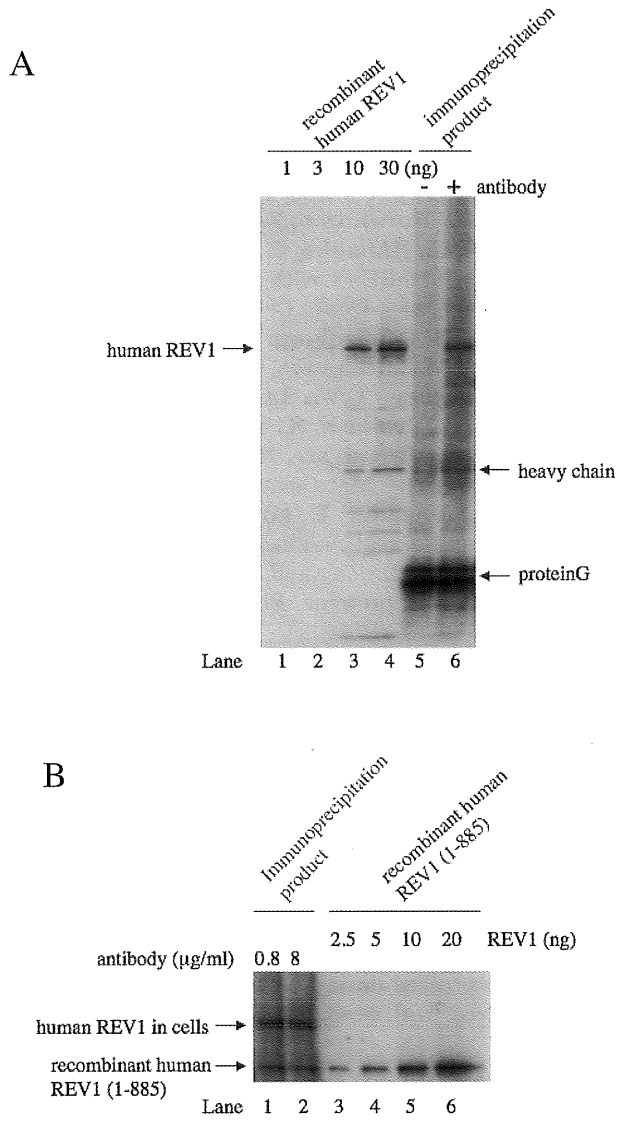

Fig. 4. Estimation of recovery of REV1 protein from HeLa cells.
(A) IP-western blotting analysis. A lysate obtained from 1×10^7 HeLa cells was subjected to IP-western blotting (lane 6). A negative control without D6 is shown in lane 5. Indicated amounts of purified recombinant REV1 were applied as standards (lanes 1-4). (B) A lysate obtained from 1×10^7 HeLa cells was combined with 10 ng of a deletion mutant of REV1, ΔC885[17]. Then the mixture was subjected to IP-western blotting under conditions of different amounts of D6 (lanes 1 and 2). The deletion mutant of REV1, ΔC885, was also applied as a standard to quantify the recovery (lanes 3-6).

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